Melatonin effects on food intake and activity rhythms in two fish species with different activity patterns: diurnal (goldfish) and nocturnal (tench)

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Running title: **Melatonin effects on diurnal and nocturnal fish**

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ABSTRACT

Melatonin has several known physiological functions, a major one being synchronization of daily and seasonal rhythms, although melatonin has also been reported to influence food intake and behavioral rhythms with varying results depending on the species. The aim of this research was to evaluate the effects of intraperitoneal melatonin injection on food intake and locomotor activity in two different fish species: goldfish (diurnal) and tench (nocturnal), under different light regimes: constant light (LL) conditions or LD 12:12, with melatonin administration at mid-light (ML), mid-dark (MD), and after a 1-hour light pulse at MD. In addition to these acute tests, in the case of goldfish we also investigated the effects of daily melatonin administration for one week. Our results indicated that acute melatonin administration significantly decreased goldfish food intake (16 to 52 % inhibition, depending on the light regime) and locomotor activity (55 to 100 %), with the chronic treatment inducing a similar total food intake inhibition that persisted for 7 days. In tench, a nocturnal fish species, acute melatonin administration at MD and ML reduced food intake (37 and 29 %, respectively), while locomotor activity was not affected at MD and slightly increased at ML. Taken together, these results indicated that melatonin reduced food intake in both species, while its effects on locomotor activity depended on the light conditions and the activity patterns of the species.

Keywords: melatonin treatment, fish, food intake, daily activity patterns

INTRODUCTION

The role of melatonin as a chemical signal for darkness that synchronizes biological rhythms is well known (Reiter, 1993). In mammals such as the Siberian hamster, the photoperiod induces seasonal changes in energy balance and body weight via melatonin secreted by the pineal gland (Morgan et al., 2003). Indeed, melatonin has been found in the gastrointestinal tissue (GIT) of all vertebrates studied (Bubenik, 2002). The role of melatonin in the GIT has been suggested to involve the regulation of bicarbonate secretion by the intestinal mucosa (Sjöblom & Flemström, 2003); and also seems to enhance the release of gastrin and stimulate exocrine pancreatic enzyme secretion (Jaworek et al., 2005). Furthermore, exogenous melatonin administration has been shown to have specific effects on a number of animal behaviors, including food intake regulation, albeit data are still scarce compared with other peptides and hormones whose involvement in feeding regulation is well established (De Pedro & Björnsson, 2001).

The effects of melatonin may differ depending on the animal's daily living habits. The indole has been shown to decrease total food intake in many diurnal species (Bermúdez et al., 1983; Bubenik & Pang, 1994; Angers et al., 2003). In the rat, a nocturnal species, there is conflicting evidence concerning the effects of melatonin on food intake, as this molecule has been reported to reduce feeding (Ishibashi et al., 1966), to have no effect (Dark et al., 1980; Wolden-Hanson et al., 2000), and even to increase food consumption (Shaji & Kulkarni, 1998; Angers et al., 2003). In the case of fish, the possible differences in melatonin action in diurnal versus nocturnal species have not yet been determined.

Aside from feeding, melatonin is also known to affect locomotor activity. Thus, it reduces the activity of all diurnal species so far evaluated (Bermúdez et al., 1983; Murakami et al., 2001; Zhdanova et al., 2001; Zhdanova et al., 2002). In nocturnal species, melatonin did

not seem to influence locomotor activity in owl (Murakami et al., 2001), whereas it slightly increased that of rats (Wolden-Hanson et al., 2000).

Although several studies have addressed the effects of melatonin on feeding behaviour and locomotor activity in a few rodents, the relationship between them is still unclear. Furthermore, the differential effects of melatonin on fish with different activity patterns (diurnal *vs*. nocturnal) have yet to be tested.

Thus, the aim of this research was to evaluate the effects of both acute and daily melatonin administration on food intake and activity rhythms in two fish species with opposite locomotor activity patterns, i. e., in goldfish (*Carassius auratus*), a fish with mostly diurnal activity (Iigo & Tabata, 1996); and tench (*Tinca tinca*), a strictly nocturnal fish (Herrero et al., 2003).

MATERIALS AND METHODS

Animals and housing

Goldfish were obtained from a local dealer (Jumipez, S.A., Murcia, Spain), and had an initial body weight of 73 ± 4 g (mean ± S.E.M.). Tench were obtained from the aquaculture centre "Las Vegas del Guadiana" (Badajoz, Spain), and had an initial body weight of 90 ± 7 g (mean ± S.E.M.). Animals were reared at the facilities of the University of Murcia, in special rooms or *chronolabs*, under constant temperature and controlled photoperiod conditions. Fish were kept in 60 liter tanks, well aerated and equipped with biological and mechanical filters, and fed a standard diet of floating pellets for pet-fish (TetraAniMin, Tetra, Germany).

Experimental procedure

Experiment 1. Effects of melatonin on food intake and locomotor activity in goldfish

These experiments were designed to investigate the effects of acute intraperitoneal melatonin administration on feeding and locomotor activity in goldfish, a diurnal fish species. Melatonin administration was performed under the following lighting conditions:

In one study, fish were first maintained under a 12:12 LD cycle with lights on at 10:00 h. During this period, food was delivered in excess (over 1.5% of body weight) at 16:00 h (ML); 30 minutes later uneaten food was removed and food intake recorded. Food was placed in 10-cm square plastic floats attached to a corner of the aquaria, to make sure food pellets remained within the feeding area. Under these conditions, we tested the acute effect of a single intraperitoneal (i.p.) melatonin injection at two different doses: 3 and 30 mg/kg. Melatonin (Sigma Aldrich Chemicals, St Louis, USA) was dissolved in a vehicle of saline solution with 1% ethanol and injected into the animals two hours before mealtime. In addition to melatonin administration, vehicle alone was injected to fish under the same conditions to control for the possible effects of fish manipulation and the injection itself.

For another experiment the photoperiod was reversed, with lights on at 22:00 h. Mealtime was maintained at 16:00 h, coinciding now with MD. After fish synchronized to the new LD cycle, melatonin (3 mg/kg) or vehicle was administered two hours before mealtime, and food intake and locomotor activity were evaluated, as described above. These procedures were performed with researchers wearing night vision goggles (D-2M Lenses mod. F24/1.4, DIPOL, Belarus), so that fish could be handled in total darkness.

The effects of exogenous melatonin were also evaluated when administered before a 1-hour light pulse at MD in order to suppress endogenous melatonin. The light pulse started 30 minutes after the injection and finished 30 minutes before mealtime, so that i.p. injection and feeding were carried out in total darkness, with the light pulse between them.

Finally, fish were kept under LL for several days, and the effects of melatonin administration on food intake and locomotor activity evaluated following a similar protocol as before.

In addition to these acute tests, the photoperiod was set at 12:12 LD and animals fed at ML. After fish acclimation to these conditions, we tested the effect of chronic melatonin administration on feeding and locomotor activity. For 7 consecutive days, fish received a daily injection of melatonin (3 mg/kg) or vehicle. After a 10-day resting period without treatment for recovery, animals were submitted to another series of Mel/Veh injections for four days, after which fish groups were crossed over and continued being treated with Veh/Mel for four additional days.

Experiment 2. Effects of melatonin on food intake and locomotor activity in tench

These experiments were designed to investigate the effects of melatonin administration (3 mg/kg) on locomotor activity and feeding of a nocturnal fish species, the tench, under two light conditions.

In the first study, fish were first maintained under a 12:12 LD cycle with lights off at 11:00 h. During this period, fish were fed (1.5% body weight) at MD (17:00 h) and, 30 minutes later, uneaten food removed using infrared goggles, and food intake recorded as described for goldfish. Melatonin was administered as a single i.p. injection (3 mg/kg) 2 hours before mealtime. As before, vehicle alone was also injected to fish to control for the possible effects of fish manipulation and injection.

Following the previous experiment, the photoperiod was reversed (lights on at 11:00 h) while mealtime was maintained at 17:00 h, thus coinciding with ML. After 2 weeks to allow fish to synchronise to the new cycle, melatonin or vehicle was administered and food intake and locomotor activity evaluated as described.

Data analysis

Locomotor activity was recorded with an infrared photocell (Omron, mod E3S-AD62, Kyoto, Japan) placed against the aquarium wall at 5 cm from the bottom. A computer connected to the photocell counted and stored the number of light beam interruptions in 10 min intervals.

Assuming that vehicle alone would modify food intake and locomotor activity, changes in these two variables were determined by calculating the difference between each fish value on the day of treatment and their mean value from previous days (n=7). Graphic representations and statistical analysis have been performed using these data.

Statistical analysis was performed using Excel® and SPSS® software. Locomotor activity records were analysed and represented with chronobiology software *El Temps* (Version 1, 192; © Prof. Díez-Noguera, University of Barcelona).

RESULTS

In goldfish, melatonin reduced food intake in all experimental groups regardless the lighting conditions (Fig. 1). Food intake variations are represented as the difference between previous food intake (base line) and the actual value recorded during acute treatment. Surprisingly, vehicle administration had opposite effects, as it increased food intake in ML and MD groups, while decreasing that of MD+L and LL groups. Average daily food intake for groups under LD and LL conditions did not change significantly and remained at 1.1 ± 0.1 % and 0.9 ± 0.1 % b.wt., respectively.

Both melatonin doses (3 and 30 mg/kg) inhibited food intake to a similar degree (16 % and 14 % inhibition, respectively), which indicated that the effect of melatonin was not dose-dependent within this range (see Fig.1, groups MEL3 and MEL30).

Under LD conditions, melatonin administration, whether at ML or MD, significantly reduced food intake (16 % and 26 %), whereas administration of vehicle alone induced a slight increase (5-8 %) (t-test, $p<0.05$). A stronger inhibition was observed in response to melatonin administration under MD+L and LL conditions (49 % and 52 %, respectively). In these cases, however, administration of vehicle did not increase but reduced food intake (34 % and 21 %, respectively).

In addition to the acute test, daily melatonin injections produced a sustained inhibitory effect (20 % reduction) on food intake for 7 days (Fig. 2A). Moreover, daily vehicle administration produced a stimulatory effect, as observed in the acute test (Fig. 1). When groups were crossed over, fish that have initially received melatonin, after being switch to vehicle alone, quickly recovered their previous food intake level (Fig. 2B). At the end of the trial, both groups recovered baseline intake values the very first day the treatment was discontinued.

In the case of tench, average food intake varied depending on mealtime and light conditions, with MD feeding (0.57 ± 0.05 %) being significantly higher than ML feeding (0.43 ± 0.05 %, paired t-test, $p<0.05$). Nevertheless, melatonin administration reduced food intake both at MD and ML (37 % and 29 %, respectively, Fig. 3). Vehicle administration also reduced food intake under both experimental conditions, but the effect was considerably stronger at ML (84 % compared to 23 % at MD). Note that the vehicle's food intake inhibition at ML was also significantly higher than that of the MEL group (paired t-test, $p<0.05$).

Goldfish displayed a predominantly diurnal activity, with 76 % of their daily activity occurring during daytime and 24 % during nighttime. Thus, baseline activities recorded (in counts per hour) were 72 during daytime and 22-23 during nighttime. Baseline activity under LL had an intermediate value (39 counts/hour) between those of daytime and nighttime when animals were maintained under a 12:12 LD cycle.

Melatonin administration modified daily activity patterns around mealtime (Fig. 4). Goldfish locomotor activity decreased after melatonin administration at ML (both with 3 and 30 mg/kg), reaching a minimum value about one hour after the injection (85-100 % of inhibition, Fig. 4A). Those fish that had received the highest dose increased their activity right after mealtime, but afterwards activity dropped again and remained lower than that of the 3 mg/kg group for the rest of the light phase.

Melatonin administration at MD produced a similar inhibition pattern (Fig. 4B), reaching a minimum (86 %) within two hours following melatonin injection, although no increase after mealtime was detected.

After a 1-hour light pulse at MD, locomotor activity increased up to 144 % in the group injected with vehicle (Fig. 4C), whereas in the melatonin-treated group it went down to 55 %. This inhibition, however, was not as strong as that seen at ML and MD (100 % and 86

%, respectively, t-test, $p<0.05$). Finally, under LL conditions, locomotor activity in both groups ran parallel with no significant differences between them at any given time point (t-test, $p>0.05$, Fig. 4D).

During daily melatonin administration, locomotor activity in response to melatonin injection was reduced for several hours after the injection, as seen during acute treatments (Fig. 5). Additionally, when groups were crossed over, locomotor activity followed a similar pattern as that observed on food intake; thus, fish that have received melatonin recovered their previous locomotor activity after being switched to vehicle alone (Fig. 5A). Curiously, total daily activity was similar in both groups (13,700 ± 4,900 *vs.* 12,400 ± 3,500 counts/day for vehicle and melatonin, respectively, t-test, $p=0.56$).

In tench, a strictly nocturnal animal, most activity (96 %) was recorded during the dark phase. Thus, baseline activities were significantly different between darkness (19 ± 4 counts/hour) and light (0.7 ± 0.2 counts/hour). As for goldfish, the activity value after an acute treatment was calculated as the count difference between the baseline activity from previous days and the activity recorded on the day of treatment.

Melatonin effects on locomotor activity, if any, were very limited. No differences whatsoever were detected at MD, and so vehicle- and melatonin-injected fish run parallel (Fig. 6A), while a slight activity increase was observed at ML (Fig. 6B) one hour after the injection (t-test $p<0.05$).

DISCUSSION

Melatonin administration had different effects on feeding and locomotor activity depending on the activity pattern of the fish species investigated. In goldfish, a mostly diurnal fish, melatonin inhibited both food intake and locomotor activity irrespective of the light regime used. In a strict nocturnal fish like tench, on the other hand, melatonin injection reduced food intake at MD and ML, but locomotor activity was not affected at MD, and a slight activity increase was detected at ML.

Melatonin can be found in the gastrointestinal tissue of most vertebrates (Bubenik & Pang, 1997), where it has been attributed a number of functions including protection of the gastrointestinal mucosa, metabolite secretion regulation and digestive motility (for a review see Bubenik, 2002). In addition, melatonin GIT levels increase in response to fasting and during feeding, suggesting a role for this indole in feeding regulation (Bubenik et al., 2000). Previous studies have reported an inhibitory effect on food intake in diurnal species (Bermúdez et al., 1983; Bubenik & Pang, 1994; Angers et al., 2003). In the present study, melatonin administration to goldfish reduced food intake regardless the lighting conditions (Fig. 1). Since goldfish can be considered a mostly diurnal species (Iigo & Tabata, 1996), even if their rhythms show a high degree of plasticity (Sánchez-Vázquez et al., 1996), our results are in agreement with those obtained in other diurnal animals.

The mechanisms mediating the inhibitory action of melatonin on food intake are still unknown. Some authors have suggested that this effect may be due to its sedative action on locomotor behavior, which has been reported in several diurnal species (Bermúdez et al., 1983; Murakami et al., 2001; Zhdanova et al., 2002). The only fish study addressing the effects of melatonin on locomotor activity was carried out in zebrafish larvae (Zhdanova et al., 2001), a diurnal species, in which melatonin added to the water inhibited zebrafish locomotor activity by 50 %. Our results in goldfish show that melatonin administration either

during the light or the dark phase inhibits the animals' food intake (Fig. 1) and locomotor activity (Fig. 4A-B). A previous goldfish study (Pinillos et al., 2001) had already reported an inhibitory effect on food intake in response to melatonin administration both at ML and MD. However, since locomotor activity was not recorded, the mechanism of action and possible relationship between feeding and locomotor activity remained unclear. Our results suggest that these effects are not necessarily related, since under constant light melatonin inhibited food intake (Fig. 1) but had no effect on locomotor activity (Fig. 4D). Most interestingly, such a discrepancy could also be observed when injections were performed before a 1-hour light pulse at MD. In this case both VEH and MEL reduced food intake (Fig. 1), but they had opposite effects on the animals' activity: vehicle stimulated and melatonin reduced locomotor activity (Fig. 4C).

In the Siberian hamster, short photoperiods induced a decrease on their total body weight (Morgan et al., 2003). Chronic melatonin treatment for 12 weeks did not modified relative food intake in rats but decreased their total body weight (Wolden-Hanson et al., 2000), an effect that was reverted when groups were crossed over. In fish, food intake inhibition has been observed in European sea bass after a chronic melatonin treatment (Rubio et al., 2004). Thus, oral administration before feeding time reduced total food intake (9 to 34%, depending on the dose used) and modified the macronutrient selection pattern during thirty days of treatment. In our study, goldfish food intake inhibition was sustained for 7 days (Fig. 2A), i.e., for the entire duration of the daily melatonin treatment, an inhibitory effect that subsided as soon as fish started to receive vehicle alone (Fig. 2B). Conversely, food intake rapidly decreased in animals that had been previously treated with vehicle when they started to receive melatonin after the crossover, as it was the case of total body weight in rats (Wolden-Hanson et al., 2000). Locomotor activity of melatonin-treated fish decreased for the duration of the treatment, subsiding at the moment when fish received vehicle alone (Fig. 5),

in a similar manner as was observed for goldfish food intake during the same treatment (Fig. 2B). This sustained effect on locomotor activity has been reported in mammals (Zhdanova et al., 2002), but has not been assessed in fish until date. Both vehicle- and melatonin-treated fish showed a similar total daily activity during chronic vehicle or melatonin administration, opposite to the inhibitory effect of melatonin on food intake and locomotor activity. In summary, the actions of melatonin on goldfish feeding behaviour are sustained in chronic treatments, as it occurs in other species of vertebrates where no development of tolerance or downregulation of these effects are observed.

In nocturnal animals, data concerning the effects of melatonin on food intake and locomotor activity are scarce and inconsistent. So far, the results in rats are contradictory (Ishibashi et al., 1966; Dark et al., 1980; Shaji & Kulkarni, 1998; Wolden-Hanson et al., 2000; Angers et al., 2003), probably due to the timing of melatonin administration. In hamsters, melatonin reduced food intake but did not affect locomotor activity (Bartness & Wade, 1985). Tench is a freshwater fish with a strictly nocturnal behaviour of locomotor activity, even under very dim light (0.3 lux, equivalent to a full moon light) (Herrero et al., 2003). As to feeding behaviour, tench also showed a strict nocturnal rhythm when they were allowed to self-feed, 98% of feeding occurring at night (Herrero et al., 2005). Furthermore, nocturnal plasma melatonin levels in tench were suppressed and reached similar values to those of ML after a 1-hour light pulse of 0.3 lux at MD (Vera et al., 2005), evincing the high sensitivity of the tench phototransducer system. The results we have obtained for tench are similar to those reported for hamsters, i.e., melatonin administration at MD reduces food intake (Fig. 3) but does not affect locomotor activity (Fig. 6A). On the contrary, when administered at ML, melatonin caused a smaller inhibition than the vehicle itself (Fig. 3), and slightly stimulated locomotor activity (Fig. 6B), which points to differential melatonin effects depending on the light conditions. This could explain the controversial results that have been

obtained in rats, where the experimental designs used, and thus light conditions and time of melatonin administration, have been very different (Ishibashi et al., 1966; Dark et al., 1980; Shaji & Kulkarni, 1998; Wolden-Hanson et al., 2000; Angers et al., 2003).

In summary, melatonin effects on tench food intake (Fig. 3) are different from those observed in locomotor activity (Fig. 6). These results, together with those obtained in goldfish under constant light, support the idea that melatonin effects on feeding and locomotor activity may be independent. These findings highlight the role of melatonin as a regulator of food intake and behavioural rhythms (feeding and locomotor activities) in fish. In goldfish, a mainly diurnal species, melatonin reduced both food intake and locomotor activity. However, in a nocturnal fish like tench, the effects of melatonin depended on the time of administration (light or dark phase), suggesting a differential action for this hormone depending on the light conditions and the way of life of the species investigated.

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FIGURE LEGENDS

Fig. 1. Food intake variations in goldfish subjected to acute administration of either melatonin (MEL) or vehicle (VEH) around mid-light (ML), mid-dark (MD) and after a light pulse at mid-dark (MD+L) under a 12:12 LD cycle, as well as under constant light (LL). MEL3 and MEL30 represent melatonin doses of 3 and 30 mg/kg, respectively. Values are expressed as mean ± SEM (n=12). Data were compared using a paired t-test. VEH/MEL3/MEL30 were subjected to one-way ANOVA, followed by Tukey *post hoc* test. Asterisks indicate statistically significant differences ($p<0.05$) between groups; n.s. indicates non-significant differences.

Fig. 2. Food intake variation in goldfish chronically treated with melatonin for seven days (2A), and following a crossover after four days of treatment (2B). Values are expressed as food intake (% body weight) differences with respect to that observed on the previous days (baseline, grey colour). The vertical dotted line indicates the beginning of treatment. Open and black circles in 2A correspond to vehicle and melatonin administration, respectively. In 2B, circles and squares are used to represent the two different fish groups, whereas open and back symbols indicate vehicle and melatonin injection, respectively. Daily mean values were compared using a t-test, $p<0.05$. Asterisks indicate statistically significant differences between groups.

Fig. 3. Food intake variations in tench injected with either melatonin (MEL) or vehicle (VEH) around MD and ML on a 12:12 LD cycle. Values are expressed as mean ± SEM (n=12). Data were compared using a paired t-test, $p<0.05$. Asterisks indicate statistically significant differences between groups.

Fig. 4. Daily locomotor activity patterns in goldfish injected with either melatonin (black circles) or vehicle (open circles) around ML (A), MD (B), MD+L (C) and LL (D). Black circles and squares at ML represent the doses of 3 and 30 mg/kg, respectively. Values are expressed as mean ± SEM (n=12). Data were compared using a paired t-test. Asterisks indicate statistically significant differences between groups (* $p<0.05$ and ** $p<0.01$).

Fig. 5. Actograms of activity from goldfish first injected with melatonin (MEL) and then crossed-over with vehicle (VEH) (5A), and viceversa (VEH-MEL) (5B). The arrows at the top represent the time of MEL/VEH administration and the mealtime. The actograms are double-plotted (time scale 48-h) for better visualization. The white and black bars at the top indicate the light and dark phase, respectively, of the LD cycle. Average daily profiles of locomotor activity during the previous phase, daily melatonin injection, and vehicle injections, have been represented on the right of the actograms.

Fig. 6. Daily locomotor activity patterns in tench injected with either melatonin (black circles) or vehicle (open circles) around MD (A) and ML (B). Values are expressed as mean ± SEM (n=12). Data were compared using a paired t-test, $p<0.05$. Asterisks indicate statistically significant differences between groups.

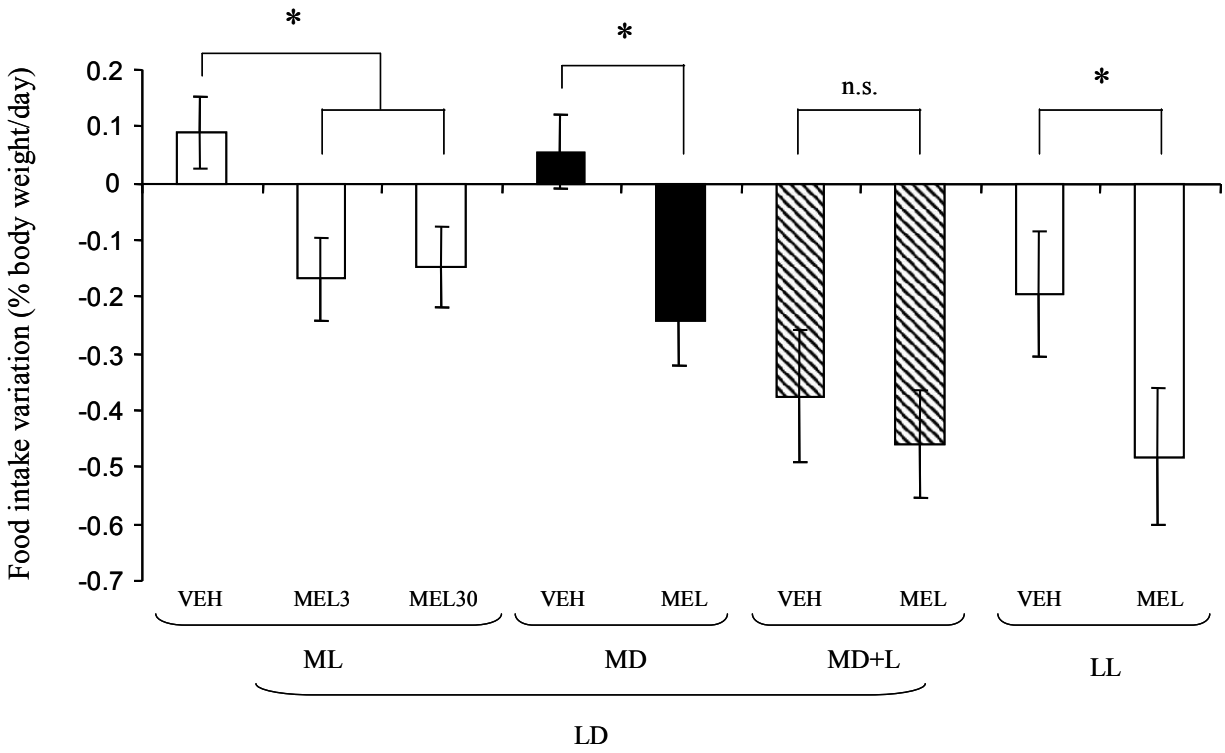

Figure 1

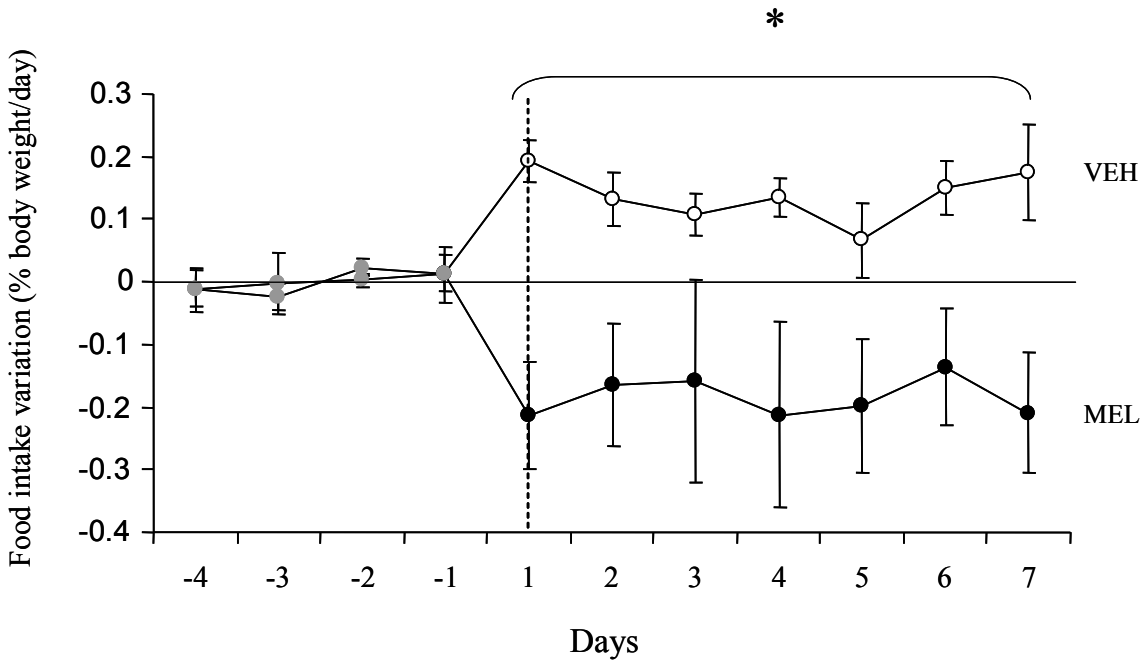

Figure 2A

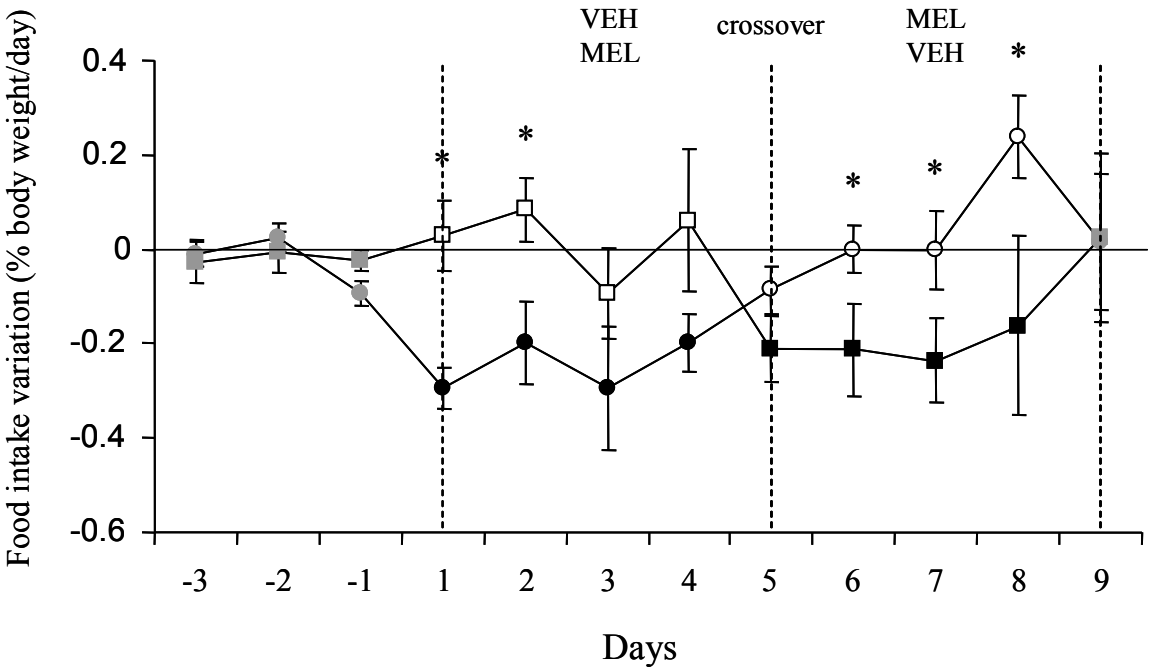

Figure 2B

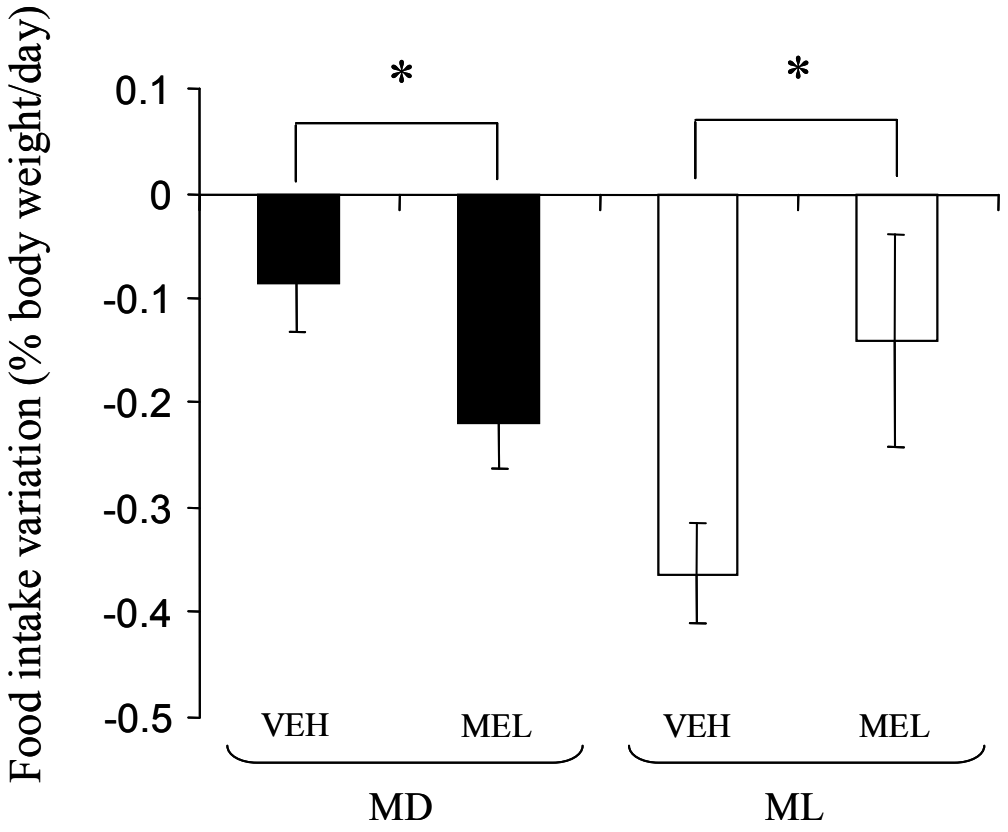
Food intake variation (% body weight/day)
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
*
*
VEH
MEL
VEH
MEL
MD
ML

Figure 3

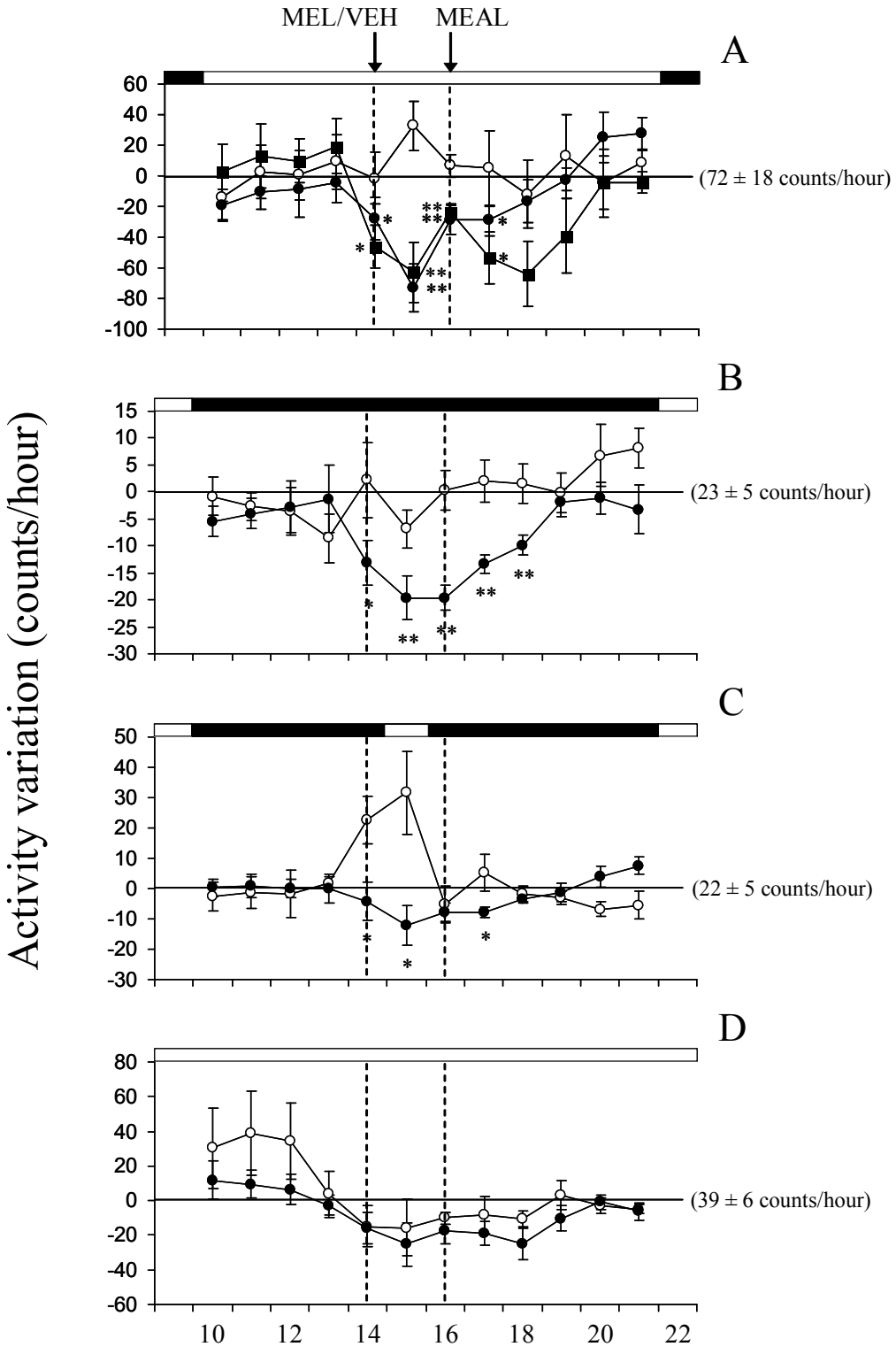
MEL/VEH
MEAL
A
B
C
D
(72 ± 18 counts/hour)
(23 ± 5 counts/hour)
(22 ± 5 counts/hour)
(39 ± 6 counts/hour)
Activity variation (counts/hour)
Time of day (h)

Figure 4

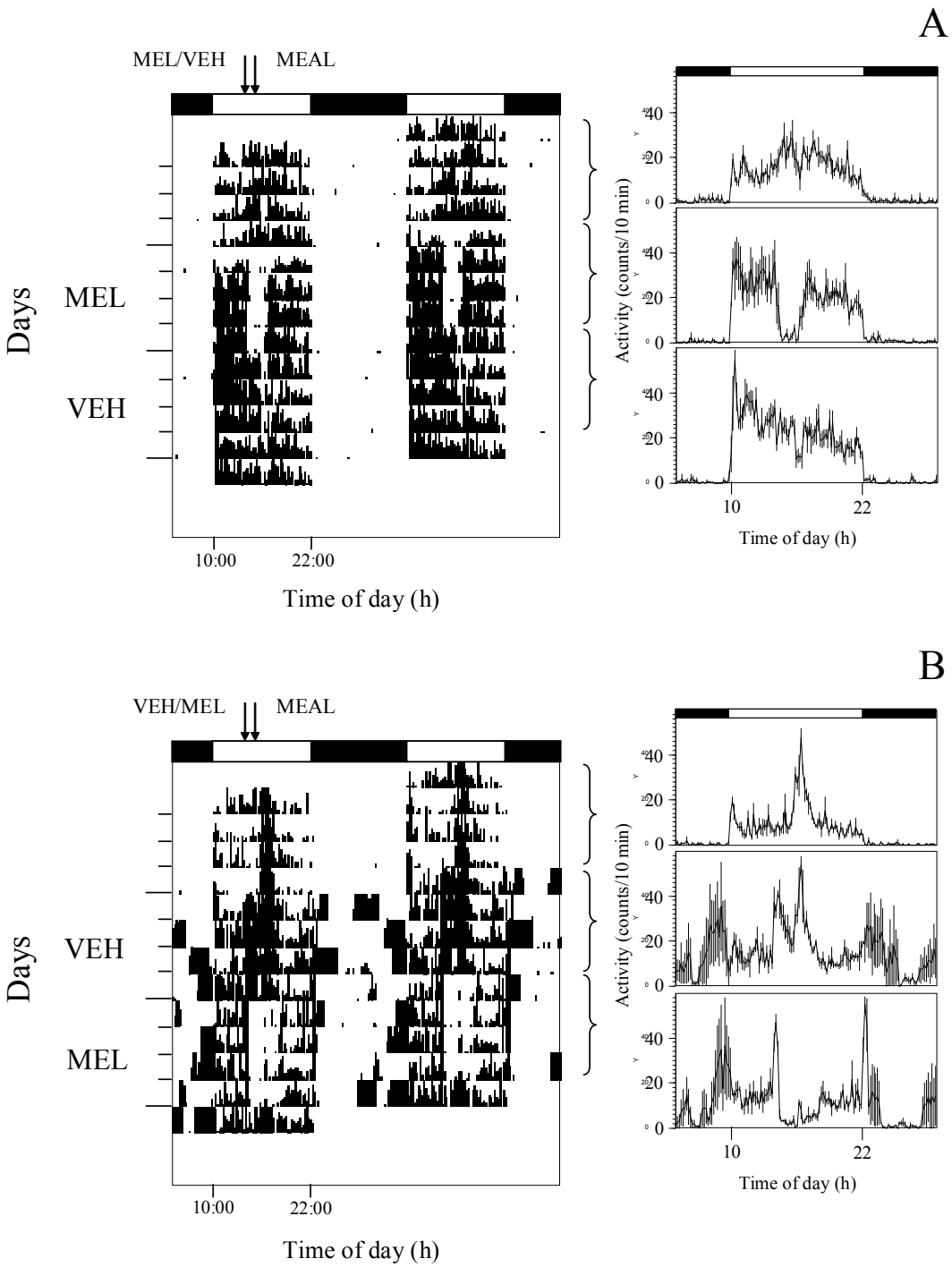
A
MEL/VEH
MEAL
Days
MEL
VEH
10:00
22:00
Time of day (h)
Activity (counts/10 min)
40
20
0
10
22
Time of day (h)
B
VEH/MEL
MEAL
Days
VEH
MEL
10:00
22:00
Time of day (h)
Activity (counts/10 min)
40
20
0
10
22
Time of day (h)

Figure 5

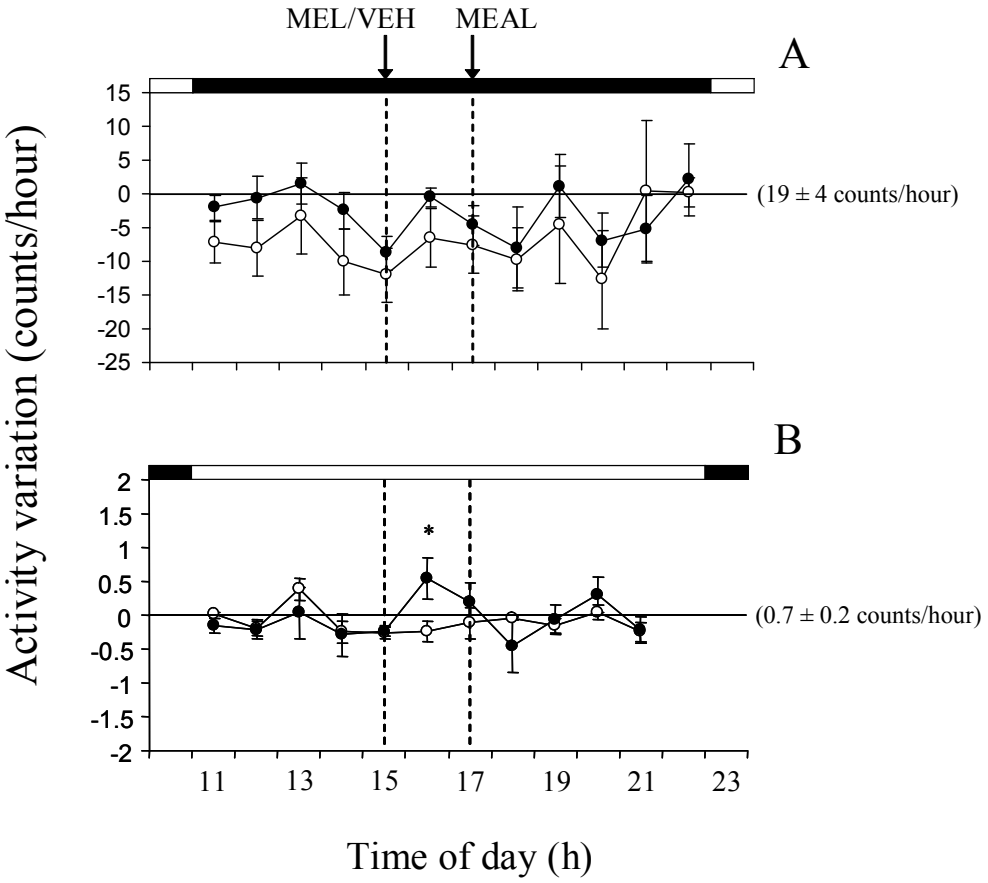
MEL/VEH
MEAL
A
B
(19 ± 4 counts/hour)
(0.7 ± 0.2 counts/hour)
*
Activity variation (counts/hour)
Time of day (h)
15
10
5
0
-5
-10
-15
-20
-25
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
11
13
15
17
19
21
23

Figure 6